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Applicant : Lieping Chen Art Unit : 1644
Serial No. : 09/649,108 Examiner : Jessica H. Roark
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Title : B7-H1, A NOVEL IMMUNOREGULATORY MOLECULE

Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF INVENTOR UNDER 37 C.F.R. §1.131

I, the inventor, declare that:

1. I am the inventor of claims 6-7, 9, 49-50, and 51 of the above-captioned patent application, as well as the inventor of the subject matter related to these claims described therein.

2. Prior to August 23, 1999, I worked in this country to complete the conception of the invention, as claimed in the application, and to reduce the invention to practice, as evidenced by the following:

A copy of a manuscript that I submitted for publication prior to August 23, 1999, is attached as Exhibit B. The other authors of the manuscript (H. Dong, G. Zhu, and K. Tamada) worked under my direction and supervision. This manuscript shows the amino acid sequence of human B7-H1 (hB7-H1) (see Fig. 1 of Exhibit B) and was ultimately published as Dong et al. (1999) Nat. Med. 5(12):1365-1369. The date of submission on the first page of the attached manuscript has been blocked out but was before August 23, 1999.

3. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

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Serial No. : 09/649,108
Filed : August 28, 2000
Page : 2

Attorney's Docket No.: 07039-220001

made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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1 Attached
for #24

Submitted to Nature Medicine

B7H, a Third Member of the B7 Family, Preferentially Costimulates Interleukin-10 Production and Increases Apoptosis of Activated T-Cells

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The costimulatory molecule B7-1 (CD80) and B7-2 (CD86) are essential components in humoral and cellular immune responses to foreign antigens¹. In the presence of antigen signal, engagement of their receptor CD28 enhances antigen-specific T cell growth, stimulates lymphokine production, and prevents activation-induced death of T-cells¹⁻⁴. Signaling of B7s through homologous receptor CTLA-4, however, deliver a negative signal to inhibit T cell activation^{5,6}. Here, we describe a third member of B7 family designated B7H. The cDNA of B7H encodes a transmembrane protein with similar tertiary structure to both B7-1 and B7-2. High abundance of the B7H mRNA is found in human placenta, heart, lung and skeletal muscle, and the counter-receptor of B7H expresses on activated CD4⁺ and CD8⁺ T-cells. B7H ligation costimulates T cell responses to polyclonal stimuli and allogeneic antigens. Unlike B7-1 and B7-2, B7H costimulation preferentially induces interleukin-10, but not interleukin-2 and interleukin-4 in T-cell cultures. Activated T-cells have increased apoptosis accompanied by up-regulated expression of Fas and Fas ligand after exposure to B7H. Our studies thus define a new costimulatory molecule that may be involved in the negative regulation of cell-mediated immune responses.

By homology search of the human cDNA expressed sequence tags (EST) database (NCBI, Rockville, MD), one EST sequence encoding a homologue to human B7-1 and B7-2 molecules was identified. The 5'- and 3'-end sequences were obtained by several independent reverse transcriptase-coupled polymerase chain reactions (RT-PCR) from a human placenta cDNA library utilizing vector sequence as primers. The full length of the gene was cloned, sequenced and named B7H (B7 homologue). The extracellular domain of B7H shares higher homology with B7-1 (20% amino acid identity) compared to B7-2 (15%) based on analysis with a McVector 6.5

software (Fig. 1B). No identical sequence was found at the Genbank database. The open reading frame of the B7H gene encodes a putative type I transmembrane protein of 290 amino acids consisting of a single immunoglobulin (Ig) V-like domain, a Ig C-like domain, a hydrophobic transmembrane domain and a cytoplasmic tail of 30 amino acids (Fig. 1A). Four structural cysteines (as labeled by a star in Fig. 1B), which are apparently involved in forming the disulfide bonds of the Ig V and Ig C domain⁷⁻¹⁰, are well conserved in all B7 members (Fig. 1B). The tyrosine residue in B7-1 (at position 87) and in B7-2 (at position 82) of the Ig V-like domain is also conserved in B7H (at position 81) (Fig. 1B). It has been reported that the motif SQDxxxELY in the Ig C-like domain (position 190-198 in B7-1 and 189-197 in B7-2, Fig. 1B) is required for the binding of B7-1 and B7-2 to their counter receptors CD28 and CTLA-4^{9,10}. However, this motif is not found in B7H (Fig. 1B). In addition, the B7HIg fusion protein, did not bind to resting T cells and Jurkat cells (Fig. 2A) despite their constitutive expression of CD28 (ref. 11 and Dong et al, unpublished observation). Further, CTLA4Ig bind 293 cells transfected with B7-1, but not with B7H cDNA plasmid (Dong et al, unpublished observation). Taken together our results indicate that B7H is not a ligand for CD28 and CTLA-4.

Northern blot analysis revealed that the expression of the B7H mRNA is abundant in heart, skeletal muscle, placenta and lung while the expression was weak in thymus, spleen, kidney and liver. There was no detectable B7H mRNA in brain, colon, small intestine and peripheral blood mononuclear cells (PBMC) (Fig. 1C). In most of the tissues positive for the B7H mRNA, two transcripts of approximately 4.1 and 7.2 kb were found (Fig.1C). By RT-PCR analysis, the transcript of the B7H was also detected in human dendritic cells, activated T cells and B cells (data not shown).

We prepared a construct containing the extracellular domain of B7H fused in frame with the Fc portion of the mouse IgG2a. The resulting fusion protein, B7HIg, was purified from the supernatants of CHO cells transfected with the construct. B7HIg was used in indirect immunofluorescence and FACS analysis to examine the expression pattern of the B7H counter-receptor (B7H-CR). Although B7H-CR is not detectable on freshly isolated PBMC of healthy donors, the expression of the B7H-CR can be upregulated on the majority of CD3⁺ T cells after incubation with anti-CD3 (Fig. 2A) or PHA (data not shown). Both CD4⁺ and CD8⁺ T cells express the B7H-CR (Fig. 2B). Similarly, anti-CD3 stimulation of the human Jurkat T cell line induced the expression of the B7H-CR while there was no detectable B7H-CR on untreated Jurkat cells (Fig. 2A). Our results demonstrate a restricted distribution of B7H-CR on activated T cells.

Despite having only ~25% amino acid sequence homology, B7-1 and B7-2 have similar equilibrium receptor binding properties and also share T cell costimulation function. To assess whether a similar activity could be ascribed to B7H, we purified T cells from human PBMC of healthy donors and stimulated them with B7HIg in the presence of the anti-CD3 monoclonal antibody (mAb). T cell proliferation from 3-day culture was determined by incorporation of ³H-TdR. B7HIg, when immobilized on culture plates, enhanced T cell proliferation up to 10-fold compared with the control Ig in the presence of suboptimal doses (5-10 ng/ml) CD3 mAb (Fig. 3A). In the absence of anti-CD3, B7HIg at the concentration up to 5 µg/ml had no effect on T cell proliferation (Fig. 3A). If the B7HIg was included in the cultures without immobilization, costimulatory effect was greatly decreased (data not shown). Consistent with this observation, inclusion of the B7HIg at levels 0.6-5 µg/ml in allogeneic MLR moderately (~2-fold) increased

the proliferation of T cells (Fig. 3B). We conclude that B7H can promote and costimulate proliferative responses of T cells to polyclonal T cell stimuli and to allogeneic antigens.

It has been reported that CD28 costimulation increases production of IL-2, IL-4, IFN- γ , IL-5, IL-13, TNF- α and GM-CSF due to increased gene transcription and mRNA stabilization^{12,13} whereas engagement of CTLA-4 by antibodies inhibit T cell proliferation, IL-2 accumulation and cell cycle progression^{5,6}. We measured levels of IL-2, IL-4, IFN- γ and IL-10 in the culture supernatants of purified T cells by sandwich ELISA after stimulation with B7HIg in the presence of anti-CD3. As shown in Fig. 4, cross-linking of T cells by the B7HIg in the presence of optimal dose of anti-CD3 at 1 μ g/ml drastically increased the production of IL-10 at 48 hrs and 72 hrs (up to 9 ng/ml) in the culture supernatants. There was no detectable IL-10 when T cells were treated by control Ig plus anti-CD3. The pattern of IL-10 production was consistent in 4 independent experiments using T cells from 4 individuals. The levels of IFN- γ also increased after 48 and 72 hrs. This increase, however, was largely due to stimulation of anti-CD3 (Fig. 4). Inclusion of B7H in the cultures can increase IFN- γ production only in some experiments at 48 hr (Fig. 4) or 72 hrs (data not shown), and the level of increase was only two-fold (Fig. 4) or less in four independent experiments. The levels of IL-2 and IL-4 were nearly undetectable (<0.15 ng/ml) or remained unchanged during the culture. Therefore, there is no consistent Th1 or Th2 cytokine pattern upon costimulation by B7H. Our data indicate that B7H costimulation selectively induces the production of IL-10.

Expression of the B7H-CR can be induced by anti-CD3 and PHA in both CD4+ and CD8+ T cells (Fig. 2), but its molecular identity remains to be elucidated. B7H-CR appears to share several features with ICOS, a recently described CD28-like molecule¹⁴. A mAb to ICOS can costimulate the growth of human T cells and induce IL-10 without secretion of IL-2 from

human T cells¹⁴. In addition, ICOS is detected on activated, but not resting T cells. However, several key functional differences between these two molecules are noted. For example, stimulation of ICOS by the mAb up-regulated many other cytokines such as IL-4, which is absent by B7H costimulation in our study (Fig. 4). Furthermore, B7H costimulation did not up-regulate expression of CD40 ligand on T cells (data not shown) whereas ICOS ligation does¹⁴. In our preliminary experiments, ICOS-Ig fusion protein did not block the binding of B7HIg to the B7H-CR on activated T cells, and there was no binding of B7HIg to ICOS-transfected 293 cells and vice versa (Dong et al, unpublished results). Our results thus suggest that B7H is not a ligand for ICOS.

Increased production of IL-10 is associated with inhibited cellular immune response, elevated Ig production and anergy of antigen-specific T cells^{15,16}. We showed that B7H-CR could be inducibly expressed *de novo* on T cells (Fig. 2A), the effect of B7H ligation on activated T cells, however, is unknown. We first examined the viability of T cells activated by high dose of anti-CD3 in the presence of immobilized B7HIg. A consistent decrease of live T cells was observed in the culture treated by B7HIg compared to that treated by control Ig as determined by typan blue in 5 experiments using T cells from different individuals. At the end of the cultures, the T cells were stained with annexin V and propidium iodide (PI) to distinguish early phase and late phase of apoptosis of T cells, respectively. The apoptotic cells in early phase shown as annexin V-positive, PI-negative cells were significantly increased to 24.8 % (SD = 8.7) by B7H costimulation compared to 14.2 % (SD = 5.7) by the control Ig in 5 experiments ($P < 0.001$). One representative experiment is shown in Fig. 5A (upper panel). Similar results were also obtained after treatment of Jurkat cells by B7H engagement (control Ig: 38.3% vs. B7HIg: 54.6%) (Fig. 5A, lower panel). The increased apoptosis was associated with upregulation of Fas

and FasL expression on B7H costimulated T cells (Fig.5B). Our results indicate that B7H costimulation increases activation-induced apoptosis of T cells in the moderate level, and the increased apoptosis is associated with the upregulation of Fas and FasL.

Fas and FasL interaction plays a critical role in activation-induced death of various types of cells, including T cells¹⁷. Our finding that both Fas and FasL are up-regulated on B7H-costimulated T cells (Fig. 5B) suggests that enhanced Fas-FasL interaction may be responsible for increased death of the T cells. It has been shown recently that lymphocytes from patients with systemic lupus erythematosus (SLE) undergo spontaneous cell death, which is associated with elevated IL-10 and upregulated FasL expression¹⁸. Blockade of FasL or IL-10 by specific mAb partially increased cell survival¹⁸. Therefore, in addition to Fas-FasL interaction, IL-10 may also play a role in cell death observed in B7H-costimulated T cells. High abundance of B7H expression is found in normal organs such as lung, placenta and muscles (Fig. 1C). Taken together with the observations that elevated IL-10 production and increased apoptosis of T cells upon B7H engagement, our results suggest that B7H may be involved in organ-specific negative regulation of cellular immune responses.

Methods

Cloning of human B7H cDNA and construction of B7H Ig fusion protein. The 5' and 3' ends of B7H cDNA were amplified by PCR from a human placenta cDNA library synthesized by SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA). The cDNA sequence of B7H was verified by DNA sequencing. The primer pairs used for the PCR were derived from the placenta library plasmid and from the expressed sequence tag (EST) clone AA292201. The full length of the B7H cDNA was amplified by PCR from the same cDNA library by specific primers and

cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA) as described^{19,20}. The B7HIg soluble protein was prepared by fusing the extracellular domain of human B7H to the CH2-CH3 domain of mouse IgG2a in the expression plasmid pmIgV (G. Zhu, unpublished data), and transfected into CHO cells. The protein in the culture supernatants was purified by a Protein G -Sephadex column (Pharmacia) and dialyzed in LPS-free PBS. Polymyxin B was also incorporated in the assays of cell proliferation and cytokine secretion to completely neutralize potential endotoxin contamination.

T cell proliferation, cytokine assays and mixed lymphocyte reaction (MLR). PBMCs of healthy donors were isolated by Ficoll-Hypaque gradient centrifugation and passed through a nylon wool column to obtain purified T cells (~85% purity). For costimulation assays, purified T cells at 1×10^5 /well in triplicate were cultured in 96-well flat-bottomed plates that were pre-coated overnight with anti-CD3 (HIT3a, PharMingen, Palo Alto, CA) in the presence of B7HIg or control Ig. To detect cytokines, supernatants were collected at 24, 48 and 72 hrs of the cultures and the concentrations of IL-2, IL-4, IFN- γ and IL-10 were determined by sandwich ELISA methods (PharMingen) according to manufacturer's instructions. T cell proliferation was determined by the addition of 1.0 μ Ci 3 H-TdR at day 2 for at least 18 hrs²¹. The incorporation of 3 H-TdR was counted by a MicroBeta TriLux liquid scintillation counter (Wallac, Finland). For MLR assays, purified T cells at 2×10^5 /well in triplicate were co-cultured with allogeneic PBMC (4000 rad-irradiated) at 2×10^5 /well in the presence of soluble B7HIg or control Ig. Four days later, the T cell proliferation was determined by 3 H-TdR incorporation.

Nucleic acid analysis. Northern blot analysis was carried out using human multiple tissue Northern blots (Clontech, Palo Alto, CA). The membrane was incubated in ExpressHyb hybridization solution (Clontech) for 30 min at 68°C. The random-primed cDNA probe was synthesized using the entire human B7H cDNA (870 bp), and was labeled using ³²P-dCTP. The hybridization was carried out for 1 hr at 68°C, washed 3 times in 2 x SSC containing 0.05% SDS, and exposed at -70°C to x-ray films.

Flow cytometry analysis. For direct immunofluorescence staining¹⁹, resting or activated PBMC or purified T cells were incubated at 4°C with 1 µg of indicated mAb for 30 min. The cells were then washed and analyzed by FACScan flow cytometry (Becton Dickinson, Mountain View, CA) with Cell Quest software (Becton Dickinson). These mAb included anti-CD3 (UCHT-1, F-0522) from Sigma, anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), anti-Fas (DX2) or anti-Fas ligand (NOK-1) from PharMingen. For indirect immunofluorescence staining¹⁹, cells were first incubated with 5 µg B7HIg at 4°C. After 30 min, the cells were washed and further incubated with FITC- (Biosource, Camarillo, CA) or PE-conjugated goat anti-mouse IgG F(ab')₂ (Southern Biotechnology Associates, Inc., Birmingham, AL) for 30 min at 4°C. The mouse IgG2a protein (Sigma) or m4-1BBIg (mIgG2a) was used as control Ig. In some experiments, Fc receptors were blocked by human or mouse Ig before incubation with FITC- or PE-conjugated mAbs.

Induction and detection of apoptosis. Human T cells or Jurkat cells at 1 x 10⁶/ml were cultured with B7HIg or control Ig at 10 µg/ml in the presence of immobilized anti-CD3 (1 µg/ml for purified T cells and 100 µg/ml for Jurkat cells). Three days later, aliquots (1 x 10⁵) of the cells

were stained by FITC-conjugated annexin V (PharMingen) at 5 μ l/test and propidium iodide (PI) (Sigma) at 5 μ g/ml for 1 hr. The samples were analyzed by flow cytometry.

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Acknowledgements.

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Figure Legends

Figure 1. (A) Predicted amino acid sequence of human B7H. The predicated signal peptide, Ig V-like domain (dash line), Ig C-like domain, transmembrane region(TM, underline) and the potential N-linked glycosylation site(*) are indicated. (B) Comparison of the extracellular protein sequence of B7H and other members of the B7 family. Alignment of B7H, B7-1 and B7-2 was obtained using the ClustalW algorithm with BLOSUM 30 matrix (MacVector, Oxford Molecular Group). Identical amino acid residues are shaded in bold and conserved residues are boxed. Cysteine residues that may be important in the forming of disulfide bond inside Ig V or Ig C domains are indicated by stars. (C) Expression of B7H mRNA. Human 12-lane multiple tissue Northern blot was hybridized with full-length B7H cDNA probe. Each lane contains approximately 1 μ g of poly A⁺ RNA.

Figure 2. Binding of B7HIg to activated T cells. Human PBMC or Jurkat cells were untreated or were activated by the plate-bound anti-CD3 mAb at 1 μ g/ml for 48 hours. (A) The cells stained with B7HIg were analyzed by flow cytometry using PE-conjugated goat anti-mouse IgG mAb (Fab')₂ (black shade). The background signal obtained with the control IgG was shown in bold open line. CD3⁺ cells were gated from activated PBMC according to the staining of FITC-conjugated anti-CD3 mAb. (B) Binding of B7HIg to CD4⁺ and CD8⁺ T cells. Activated PBMC, generated by plate-bound anti-CD3 as described in (A) were double stained with FITC-conjugated anti-CD4 or CD8 mAbs, and B7HIg or control Ig followed with PE-conjugated anti-mouse Ig mAb. The numbers indicated the percentages of B7HIg or control Ig binding cells in CD4⁺ or CD8⁺ cells. Results of a representative experiment out of three are shown.

Figure 3. Costimulation of T cell growth by B7HIg. (A) Co-stimulation of T cell proliferation. Nylon wool-purified T cells at 1×10^5 /well were cultured in the presence of plate-bound anti-CD3 at the indicated concentrations and 5 $\mu\text{g}/\text{ml}$ of plate-bound B7HIg or control Ig for 3 days. (B) Effect of B7HIg in MLR. Purified T cells at 2×10^5 /well were cultured with irradiated allogeneic PBMC at 2×10^5 /well in the presence of soluble B7HIg or control Ig at the indicated concentrations for four days (see methods). Proliferation of T cells was determined by incorporation of ^3H -TdR during the last 18 hrs of the cultures. Results of a representative experiment out of three are shown.

Figure 4. Cytokine secretion of primary T cells by B7H costimulation. Nylon wool-purified T cells at 1×10^5 /well were stimulated by plate-bound CD3 at 1 $\mu\text{g}/\text{ml}$ in the presence of 10 $\mu\text{g}/\text{ml}$ of either B7HIg or control Ig. Supernatants were collected at 24, 48 and 72 hr. Indicated cytokines were detected by sandwich enzyme-linked immunosorbant assays (ELISA). Results of a representative experiment out of four are shown.

Figure 5. Activation-induced cell death, Fas and Fas ligand expression in B7H-costimulated T cells. Nylon wool-purified T cells or Jurkat cells at 1×10^5 /well were cultured for 72 hours in the presence of plate-bound anti-CD3 (1 $\mu\text{g}/\text{ml}$ for purified T cells, and 100 $\mu\text{g}/\text{ml}$ for Jurkat cells) and 10 $\mu\text{g}/\text{ml}$ of B7HIg or control Ig. (A) Early apoptotic cells are defined by the annexin V $^+$ and propidium iodide (PI) $^-$ (see Materials and Methods). (B) Expression of Fas and FasL in B7H-costimulated T cells. Nylon wool-purified T cells were treated as shown in (A), and were subsequently stained with FITC-conjugated anti-Fas mAb or purified anti-FasL mAb followed by FITC-conjugated goat anti-mouse IgG (Fab') $_2$. Numbers on the bar indicated the % of

positive cells compared with non-specific staining control. Results of a representative experiment out of four are shown.

Figure 1

A

Signal peptide

1 MRIFAVFIFMTYWHLLNAFTVTVPKDLYVVEYGSNMTIECKFPVEKQLDL

Ig-V-like

51 AALIVYWEMEDKNIIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQ

101 ITDVKLQDAGVYRCMISYGGADYKRITVKVNAPYNKINQRILVVDPVTSE

Ig-C-like

151 HELTCQAEGYPKAEVWTSSDHQVLSGKTITNSKREEKLFNVTSTLRIN

201 TTTNEIFYCTFRRLDPEENHTAELVIPELPLAHPPNERTHLVILGAILLC

TM

251 LGVALTFIFRLRKGRMMMDVKKCGIQDTNSKKQSDTHLEET

Figure 1

B

B7H 30 V E Y G S N M T I E C K A P V E K Q L D I A A L I V A Y V E M
 B7-1 43 K E V A D E S C G H N V S - V E E L A Q T R I M V O K
 B7-2 30 A Y F N E T A D L P C Q I E A N E Q N Q S L S E L V W F V Q D

60 E D I K N I I Q P V H G E E D - L K V Q H S S Y R Q R A R L L
 69 E K K M V S L T M M S G D M N - - - I W P E X K N E R I F D
 60 Q E N L V I N E V Y L G K E K F D S V H E K Y M G R I S F D

89 K D Q L S L G N A A I Q H T D V K L Q D A G V Y R C M I S Y ★
 95 I T - - - N N L S I V V L A R P S D E G T X E G V V V L K
 90 S - - - D S W T I R L H N L Q I K D K G L Y Q C I T H H

119 G G A D Y K R - - - I T V K V N A P Y H K I N Q R I L V V
 121 Y E K D A F K R E H L A E V T I S V K A D E P T P S I S D F
 115 K K P T G M I R I H Q M N S E L S V A N F S Q P E I V P I

145 D P V T S S E H E L - - - E C - Q A E G Y P K A - E V I W T S S ★
 151 E I P T S N I R R - - I I C S T S G G F P E P - H L S W I E N
 145 S N I T E N V Y I N L T C S S I H G Y P E P K K M S V L L R

171 D H Q - - - V I S G K T I I T I N S K R E E K L F N V I S T I L
 179 G E - - - E L N A I N I I V S Q D P E T E L Y A V S S K I L
 175 T K N S T I E Y D G I M Q K - S Q D N V I E L Y D V S I S I

198 R I N - - - T I I N E I E Y C T F R R L D P E E N H T A E L ★
 205 D F N - - - M I I N H S F M C L I K Y G H L R V N - - Q T E
 204 S V S F P D V I S N M T I F C I L E T D K T R L L S - S P E

225 V I P E L P L A H P P N E R T
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 233 S I E L E D P Q P P D H I P

C

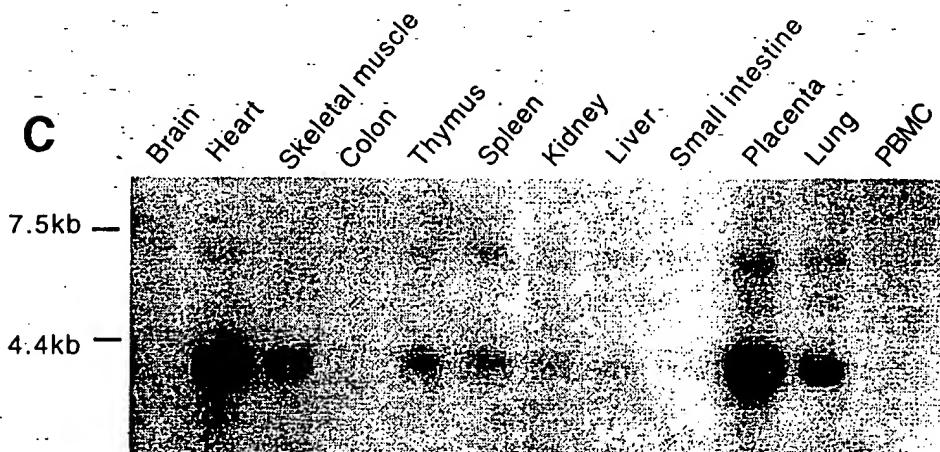


Figure 2

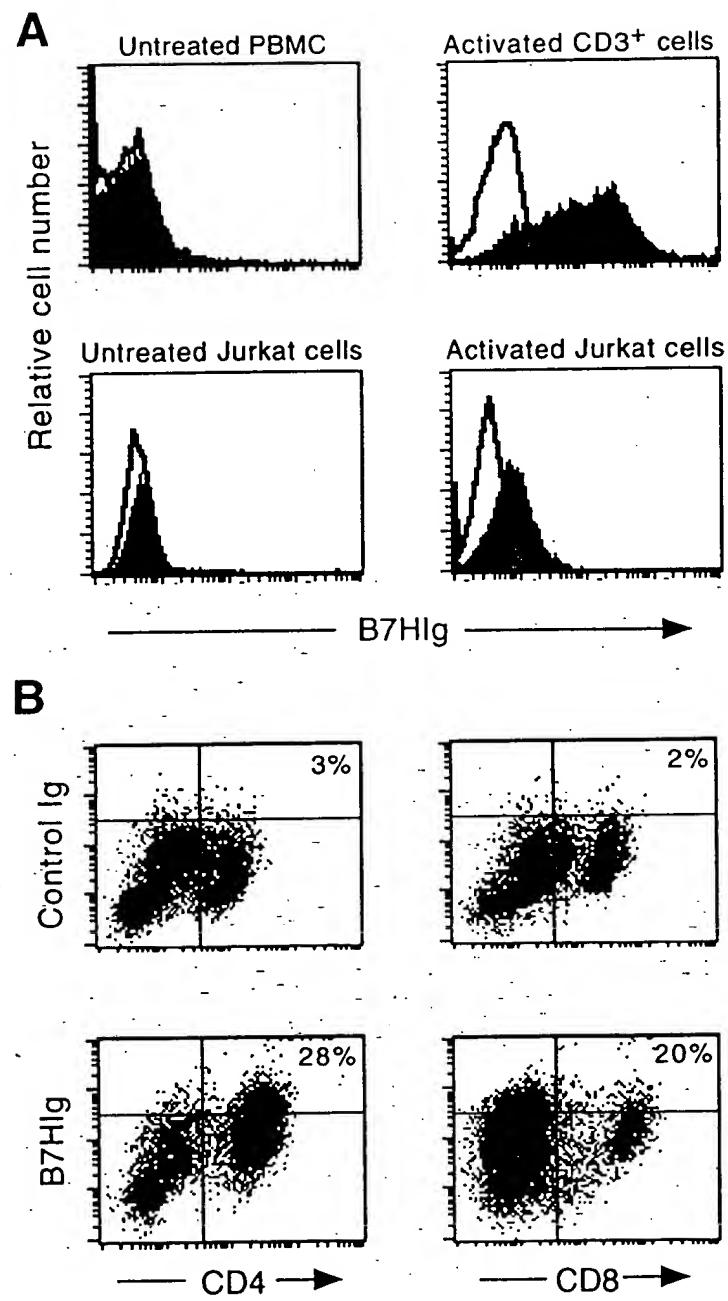


Figure 3

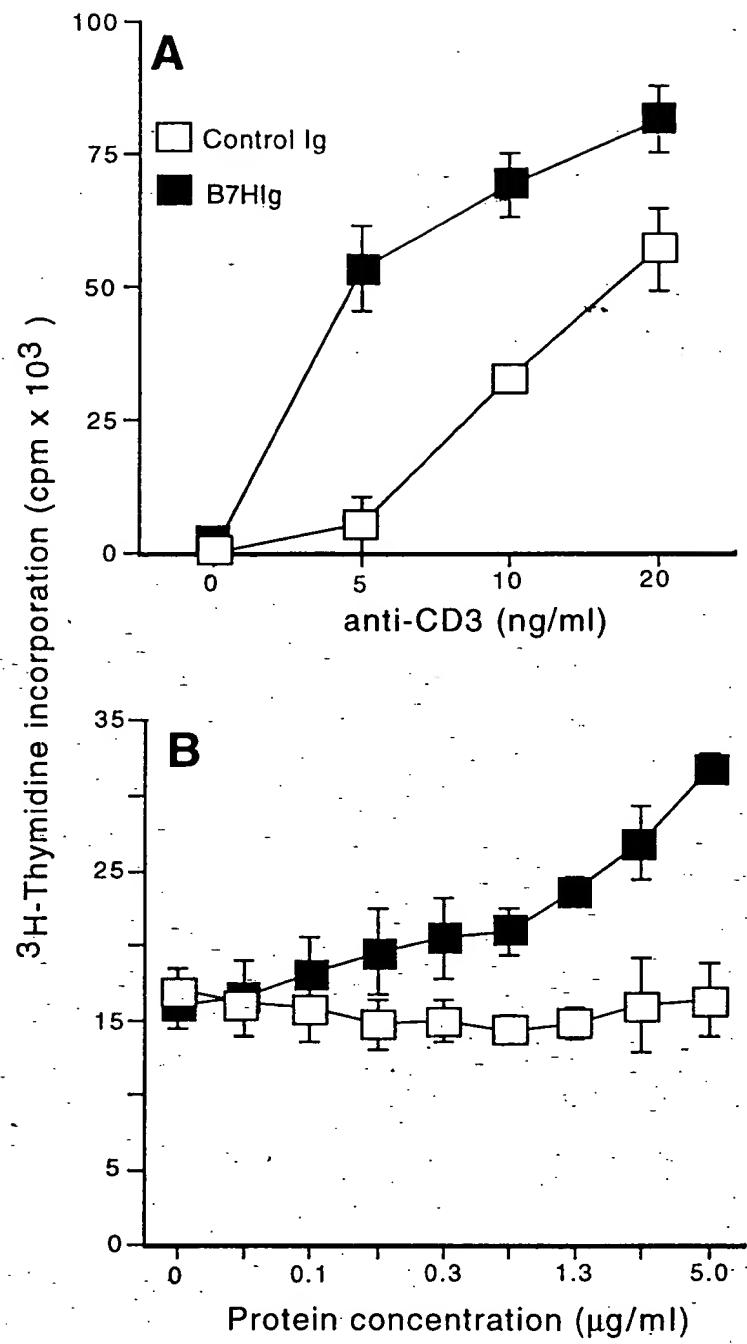


Figure 4

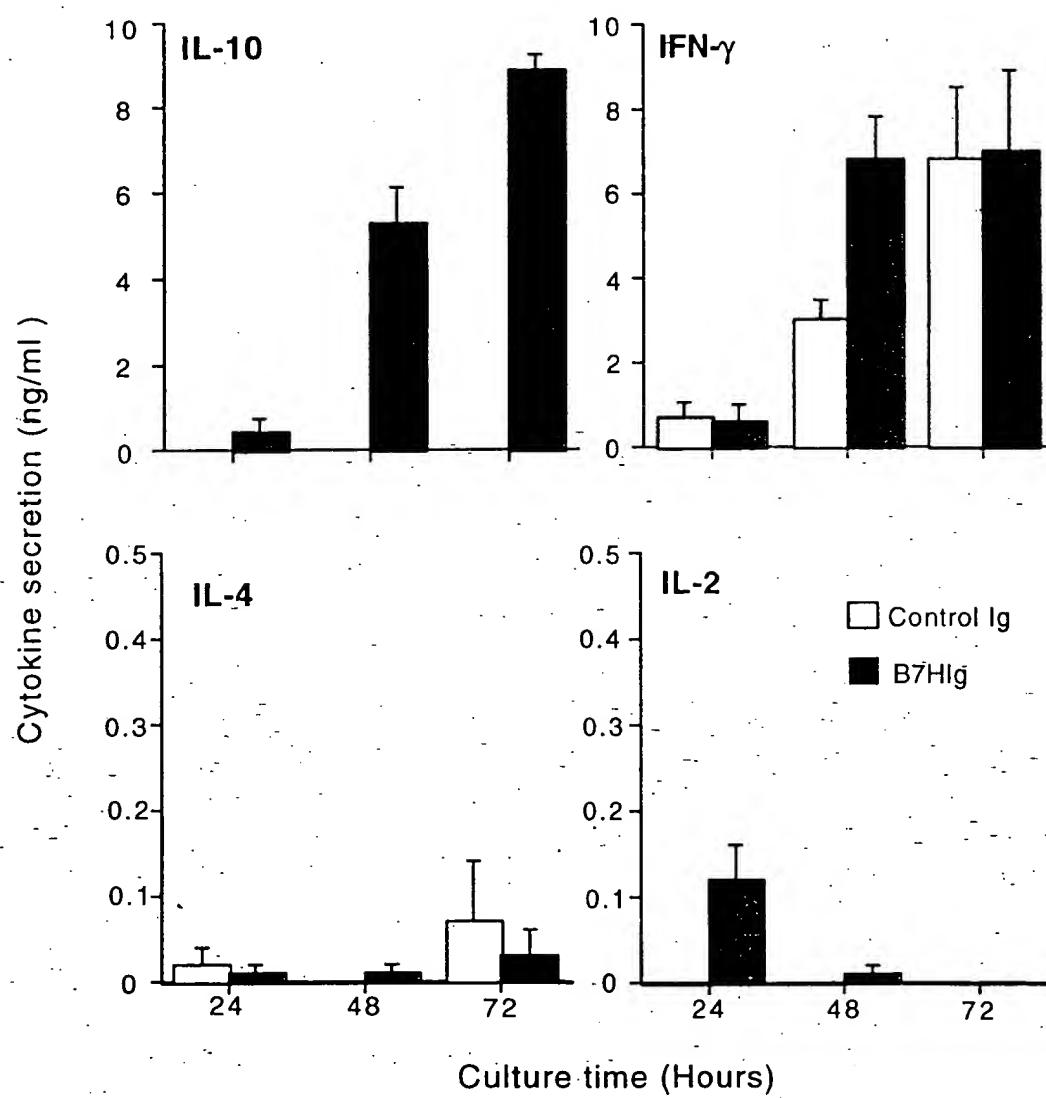


Figure 5.

